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The Relationship Between Bacillus Amylovorus and Leaf Tissues of the Apple

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The Relationship between *Bacillus amylovorus* and Leaf Tissues of the Apple[1]

JULIA MOESEL HABER

SINCE the earliest days of American fruit culture much investigational work of a general nature has been done upon "fire blight." Following the discovery of the causal organism, *Bacillus amylovorus*[2] by Burrill in 1881 (6), few attempts have been made to study the migration of the bacteria in the host tissues of the pomes and the leaves have been treated only in a casual manner.

Literature Review

Concerning experiments with leaf infection by *Bacillus amylovorus*[2] Burrill (6) says, "the most perfect and apparently healthy leaves were selected, and an account kept as to the application of *B. amylovorus* to the upper and under surface. No certain infection followed. . . . the experiments do tend to show that the virus is harmless on the outside of the epidermal surfaces. Neither does it appear to gain access to the inner tissues through the stomata."

According to Arthur (2) repeated attempts to convey the disease by inoculating the leaves resulted in failure except for a partial success when young leaves were used. Leaves are the last to succumb to the disease; death of leaves on a diseased branch is due to cutting off of sap supply.

Waite (12) states "while the bacteria themselves rarely kill the leaves at most only occasionally attacking the stems and midribs of youngest ones, all the foliage on the blighted branches must, of course, eventually die."

Snyder (10) reports that the leaves do not take the blight when inoculated naturally, the germ appears to be confined to the branches, the leaves dying only when their water supply has been cut off.

E. F. Smith (8) notes that the dark lines run out along the petiole, midrib, or side veins, the infection, however, migrating from stem up into the petiole.

Recently Nixon (7) made an extensive review of the literature on *B. amylovorus* and its relation to host tissues emphasizing the conclusions made by Burrill (5, 6), Bachman (4), Waite (12), and others regarding the path, method, and rate of migration of the bacteria

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[2] In Bergey's Manual of Determinative Bacteriology, revised edition (1925), this organism is known as *Erwinia amylovora*, but in this paper the author has used the former nomenclature, *Bacillus amylovorus*.

and their relation to the host tissues. Nixon also found that the life cycle of *B. amylovorus* in the stem comprises two phases: a vegetative and a stage of pseudo-fructification. In the former stage, the zoogloeae migrate through intercellular spaces of the cortex, forming schizogenous cavities. In the latter phase the migration becomes intracellular, forming lysigenous cavities and terminating in the formation of cysts. The cysts represent the winter condition of the bacteria.

Because the pertinent observations have been so few, it seemed that investigations on the foliage would be profitable. Two questions have arisen. First, can the leaf be the portal of infection? Second, if so, what is the course of the pathogen in its migration? These questions led to an investigation of the movement of *B. amylovorus* in the leaf, and of the relationship between host and parasite.

The Morphology of the Leaf

The apple leaf is comprised of abundant mesophyll, veins, and epidermis. Adjacent to the upper epidermis is the palisade mesophyll which is composed usually of two, but often of three layers of elongate, more or less cylindrical cells. These merge into the spongy mesophyll, the cells of which are short, irregular in shape, and loosely arranged. The mesophyll tissue, especially the spongy mesophyll, is honeycombed with connected intercellular air spaces of varying sizes. The stomatal chambers are fairly large. The epidermis consists of cells, rectangular in cross section, protected outwardly by a firm cuticle. The lower epidermis is broken by slightly projecting stomata. The veins are composed of collateral bundles.

Materials and Methods

Only the young leaves on shoots or water sprouts of King, Delicious, Stayman, and Rome Beauty varieties of apples grown in the greenhouse were used for this study. The shoots or water sprouts were over five inches in length. One hundred and fifty-eight inoculations were made in series, five series of which were made in May and four in December. The bacilli were introduced into the young leaves by means of a microlepidopterous needle, producing a small puncture from upper to lower epidermis. These punctures involved various parts of the leaf.

In order to study the relation of the organism to the host tissue it was of utmost importance to note the early stages of this bacterial invasion of the tissue. Consequently material was taken at various time intervals after inoculation—immediately afterward, 15 minutes later, 30 minutes after inoculation, and thereafter at intervals of 30 minutes to the sixth hour; then at one hour intervals to the twelfth hour following inoculation, and finally after 24, 48 and 72 hours.

For comparative study, checks were selected of similar leaves punctured with sterile needles and of leaves with normal tissues. The material was fixed in either Flemming's weak, Petrunkevitch's or alcohol-formalin-acetic solution, and imbedded in paraffin by the usual method. Both transverse sections and sections parallel to the surface of the blade were made. Sections were cut 10 microns thick, mounted, and stained in a modified Flemming's triple stain.

Observations

The Symptoms of Infection—The first phase of the work undertaken was to determine whether the leaf can become the portal of infection. The usual macroscopic symptoms, such as browning in the region of the puncture, and the characteristic blighted appearance were evident. Eight out of nine of the series, or about 94 per cent of the inoculated leaves, gave evidence of susceptibility to fire blight.

The Portal of Infection—Microscopically there was much evidence of infection. In the process of inoculation, no effort was made to control the number of bacilli for each dosage. In all cases, the inoculating needle penetrated the blade from epidermis to epidermis. This gave all leaf tissues an equal chance of infection. In material taken about 15 minutes to one hour after inoculation, single masses of bacteria were evident in the mesophyll bordering the wound (Fig. 1). The masses contained at most 6 or 7 bacteria "in situ." The spongy mesophyll is the most susceptible tissue of the leaf. In many instances, bacteria, distributed along the path of the needle in the palisade region, migrated toward the spongy mesophyll and entered the leaf tissue in the area where the spongy mesophyll borders on the palisade layer. The spongy and not the palisade mesophyll is the portal of infection (Fig. 2).

The Formation of Zoogloeae—Preparation of material taken 15 minutes to one hour after inoculation disclosed the presence of bacteria in the wound, in numbers approximately equivalent to those released when making the puncture (Fig. 1). In one and one-half hours, groups of bacteria were observed in the margin of the puncture, adjacent to the lacerated cells. These bacilli were imbedded in a homogenous jelly-like matrix, irregular in shape, but with a uniformly even and unbroken surface and with a clearly defined border. This gelatinous mass in the zoogloea (Fig. 2, z.).

The Zoogloeae—In the early stages of development the zoogloeae stain is a light blue; in later stages a darker shade, indicating that the matrix becomes denser and is of a more coherent consistency. In the oldest stages it appears a deep red, due to the fact that nothing but bacilli, which stain red, are visible in the mass. The advancing

tips of the zoogloeae assume various shapes and sizes corresponding to the intercellular spaces which they occupy. Adjacent to palisade cells in the margin of the puncture the zoogloeae become narrow and elongated (Fig. 2). The tip end is blunt. When the zoogloeae penetrate the spongy mesophyll they become short, quite broad, and irregular in contour. The advancing tip assumes a scalloped edge (Fig. 9). Each rounded projection forms a new pseudopodium which ultimately advances in a new direction.

There seems to be a close relationship between the parasite and its host, for the form and the size of the zoogloeae are entirely dependent upon the intercellular spaces of the infested tissues. As an intercellular space is invaded the matrix proceeds first, with a few bacilli acting as a vanguard. Gradually the organisms stream and orient themselves along the periphery of the matrix (Fig. 8). The branches of the zoogloeae nearest the point of infection become filled with numerous bacteria so as to apparently obliterate the matrix from view.

Bacillus amylovorus—The bacteria are imbedded in the zoogloeae. They are rod-shaped (Figs. 8, 9). They may be isolated, arranged in pairs, united more or less end to end to form zig-zag chains, or they may be massed. Each bacillus is surrounded by a hyaline envelope of uniform thickness (Fig. 9). The inner margin of this envelope conforms to the outline of the bacillus, while the outer surface of the "halo" is the surface of the surrounding matrix. When bacteria are so numerous as to obscure the matrix, the hyaline envelopes appear less distinct, often being invisible. When 3 to 6 bacteria lie in close proximity a single common envelope may surround the entire group. The bacteria multiply by fission. The reproduction is most active in the branches of the zoogloeae nearest the portal of infection and continues until the intercellular spaces become gorged with bacilli (Figs. 3-7). Thus, in the advancing zoogloeal tip there are few well-defined bacteria with definite hyaline areas surrounding them (Fig. 9). In the more remote portions definite outline of individuals are obliterated by huge masses of bacteria.

The Path of Migration—The zoogloeae always form on the margin of the puncture among the lacerated cells, but rarely fill the cavity, or obstruct the opening caused by the needle. From the portal of infection, the spongy mesophyll bordering the puncture (Fig. 2), the zoogloeal mass proceeds in all directions into the healthy tissue. By pseudopod-like expansions the original zoogloeae invade the large intercellular spaces which offer little resistance to their progress. By the formation of new tips the zoogloeae ramify from the area of infection, forming an anastomosing system, which rapidly fills the connected intercellular spaces. Ultimately a single host cell may be surrounded entirely by invading zoogloeae.

If the inoculating material passes through a vein, the resultant infection seems to spread most readily in the surrounding vein parenchyma. In the region of the midrib the zoogloeae are developed first in the vein parenchyma, about four or five cells beneath the epidermis. From this region migration proceeds in all directions, radially, tangentially, and longitudinally. This migration accords with the path in the stem as reported by Nixon (7). Migration eventually is toward the epidermis. In one observed instance the zoogloeal tip penetrated the sub-stomatal air chamber within 24 hours after inoculation. The advancing tip was exceedingly broad and, no doubt, in a few hours more might have proceeded through the stomatal opening (Fig. 9), and appeared as the typical exudate over the lower epidermis. Thus active invasion is confined to the intercellular spaces in the spongy mesophyll, and the parenchymatous layers sursounding the veins. Great care must be exercised in not confusing the dense, deeply stained cell contents of the phloem with masses of bacilli.

The Rate of Migration—Evidence of zoogloeal invasion was first observed at the edges of the wound. In material secured about one hour after inoculation, zoogloeae had traveled from the edge of the puncture in the spongy mesophyll, on the average, a distance of .0195 mm.; at the end of six hours .08763 mm. In eight hours the zoogloeae migrated .381 mm. and .5715 mm. at the end of 12 hours. In 24 hours the pseudopod-like structure had traveled tangentially a distance of 1.4 mm. in the parenchyma of the midrib, involving three-quarters of the circumference of the midrib on the lower side of the blade.

The most rapid migration occurred in the early stages of infection, between 6 and 8 hours after inoculation. In the fall series the zoogloeal movement was much slower and more feeble than in the spring series. The most rapid migration occurred within 20 to 24 hours after inoculation.

It is difficult to give accurate rates of migration, for zoogloeae having once reached the spongy mesophyll region, will migrate in various directions. These rates were obtained by measuring the length of the zoogloeae traveling parallel to the surface of the blade of the leaf.

The Effect on the Host Tissues—When the inoculating needle bearing a quantity of *B. amylovorus* penetrates young leaves, a considerable number of cells of various tissues are destroyed. Every gradation of injury from a slight tear to complete destruction occurs in the cells on the margin of the wound (Fig. 1). Those cells im-

mediately bordering the edge of the needle puncture have lost all semblance of organization and appear thick and amorphous. They are stained a golden brown by orange G. The injury becomes less pronounced the farther the cells are removed from the margin of the wound.

Because of the necrosis of the cells bordering the puncture, the resulting wide spaces between the cells are open to the unimpeded migration of the zoogloeae. These migrate from the region of wounded cells into that of the healthy tissue. Here the zoogloeae approach and pass by host cells without the least visible effect upon them. Numerous host cells are surrounded almost completely by bacilli which produce no obvious change in them (Fig. 3).

Depending upon the condition of the host cell, the first apparent effect is a slight plasmolysis (Fig. 4). This plasmolysis proceeds quite slowly as is evidenced by the gradual irregular recession of the protoplast from its cell wall. Correlated with plasmolysis is the gradual breaking down of the starch granules and the destruction of the plastids. Eventually the protoplast collapses completely, all cellular organization having been destroyed (Fig. 5).

In the meanwhile the zoogloeae, filled with masses of bacteria, have distended the intercellar cavities surrounding the host cells. Undoubtedly, the pressure upon these cells, exerted by the prolific multiplication of the bacteria, causes the cell walls to collapse (Fig. 6). Eighteen hours after inoculation, the protoplast has diminished in size and the walls have become much distorted by the pressure exerted upon them by the advancing and increasing bacterial masses. The result is a larger or smaller schizogenous cavity formed by the splitting and separating of the cell walls (Fig. 6).

At the end of 24 hours, several small schizogenous cavities form an anastomosing system. Gradually the branches become united, forming larger cavities which become filled with bacilli. In many instances these schizogenous cavities completely take the place formerly occupied by the spongy mesophyll (Fig. 7). There is no indication of the complete dissolution of the cell walls or the cell contents of the host cells thus invaded. Bacterial mass pressure pushes the remaining protoplasts toward the epidermal layers. There is no evidence that the bacteria enter the cells and cause complete disintegration of the protoplast.

Discussion

Burrill found upon microscopical and other examination that leaves upon healthy shoots may perish with the disease. During dewy nights he observed that infected leaves became smeared with an exuding virus which upon drying looks like varnish.

Arthur found that leaf inoculation was a partial success when young leaves were tried.

The consensus of opinion of earlier investigators, (Arthur, Waite, Snyder, Smith), was that the leaves of the apple do not take the blight when inoculated naturally. The presence of bacilli in the leaf tissue indicated infection from the stem to the blade, through the petiole.

Artificial inoculation of the leaf itself, to determine the possibility of the foliage as a portal of infection, was overlooked. In the belief that leaf tissue was infected through the stem, further investigations along this line evidently were regarded as valueless. No work had been done on the cellular relationship between parasite and host.

This investigation shows that fire blight may be incipient in the young leaves of water sprouts, when artificially introduced. The causal organism was traced from its incipiency in the needle puncture to its presence in the formation of schizogenous cavities. Emphasis also was placed on the movement of the zoogloeae, and the behavior of the bacilli during migration.

It has been observed further that bacilli were distributed along the path of the needle puncture, but that not all tissues were infected. Though zoogloeae were formed alongside the injured palisade cells, the actual entrance of the zoogloeae into the leaf tissue was in the spongy mesophyll or the vein parenchyma. This may indicate that there is a distinct relationship between host and parasite. The spongy mesophyll and vein parenchyma are the most susceptible tissues. The high food content of these cells may be the basis of selection for bacterial activity.

When zoogloeae penetrate sub-stomatal air chambers, their advancement soon becomes limited. The stomatal opening serves as a means for the release of the pathogen in the form of an exudate.

The early condition of the bacteria, the formation of zoogloeae, the path and manner of migration of the zoogloeae parallel the results found by Nixon in his study on the apple stem. These results differ from his in that in the leaf *B. amylovorus* forms only schizogenous cavities and exists only in the vegetative stage. There is no evidence of intracellular invasion even in material 72 and 96 hours after inoculation.

Conclusions

1. Under artificial conditions young apple leaves can be the portal of infection for fire blight organisms.

2. The spongy mesophyll of the blade or the parenchyma of the vein in the leaf is the infection-court.

3. The bacilli in the form of zoogloeae migrate in the spongy mesophyll. Movement is intercellular.

4. The nature of the zoogloeae is closely correlated with its environment.

5. The cellular effects on the host tissues include plasmolysis of the protoplast, the collapse of cell walls, and the separation of contiguous cell walls to form schizogenous cavities.

6. *B. amylovorus* multiplies by fission in the zoogloeae.

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Description of Illustrations

Figures 1-9

Fig. 1. A cross-section of a young leaf in the region of a vein showing the result to the cells of the needle puncture, and also the bacteria released in the process of inoculation. P-palisade layer; s-spongy mesophyll; v-parenchyma of vein.

Fig. 2. Cross-section of leaf showing the formation of zoogloeae (z) along the palisade layer at the margin of the puncture and their entrance into the intercellular spaces of the spongy mesophyll, eight hours after inoculation. Many branches to advancing tips.

Fig. 3. Cells from cross-section of parenchyma of midrib showing a single healthy host cell surrounded by zoogloeae.

Fig. 4. Cells from cross-section of vein parenchyma showing plasmolysis of host cells.

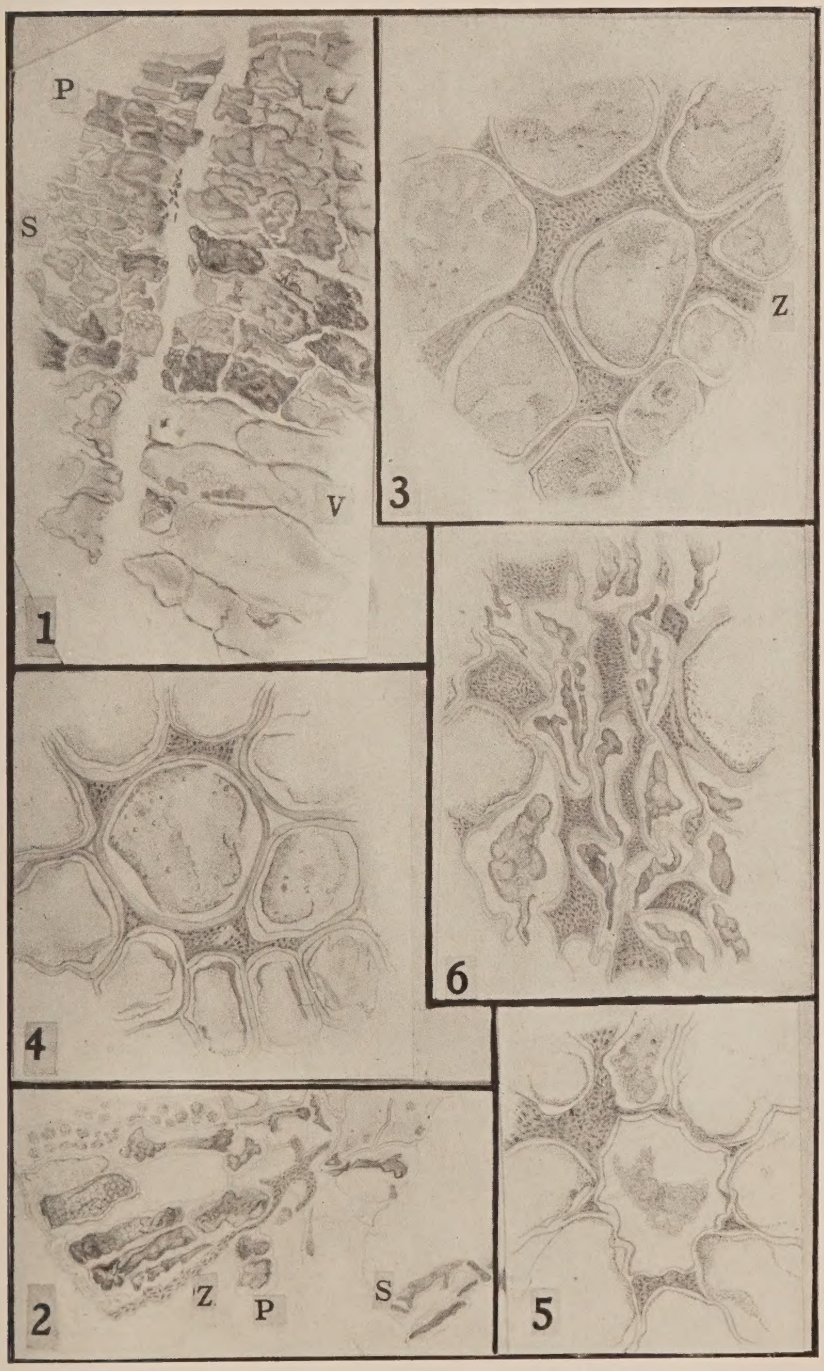
Fig. 5. Parenchyma cells from the cross-section of a vein showing the reduction of the protoplast and the collapse of cell walls.

Fig. 6. Cells from the cross-section of blade showing the death of the host cells and the formation of small schizogenous cavities and the anastomosing zoogloeae.

Fig. 7. A large schizogenous cavity in the spongy mesophyll; the death of host tissues.

Fig. 8. A streaming of the bacterial mass in the zoogloeae when invading an intercellular space. Note the various directions of migration.

Fig. 9. A zoogloea penetrating the sub-stomatal air-chamber of midrib as well as the surrounding intercellular spaces, 24 hours after inoculation. The advancing margin is broad and gives rise to at least three projections, each migrating in a different direction. The bacteria are arranged in groups, or in zig-zag chain. Hyaline areas are visible about the bacilli.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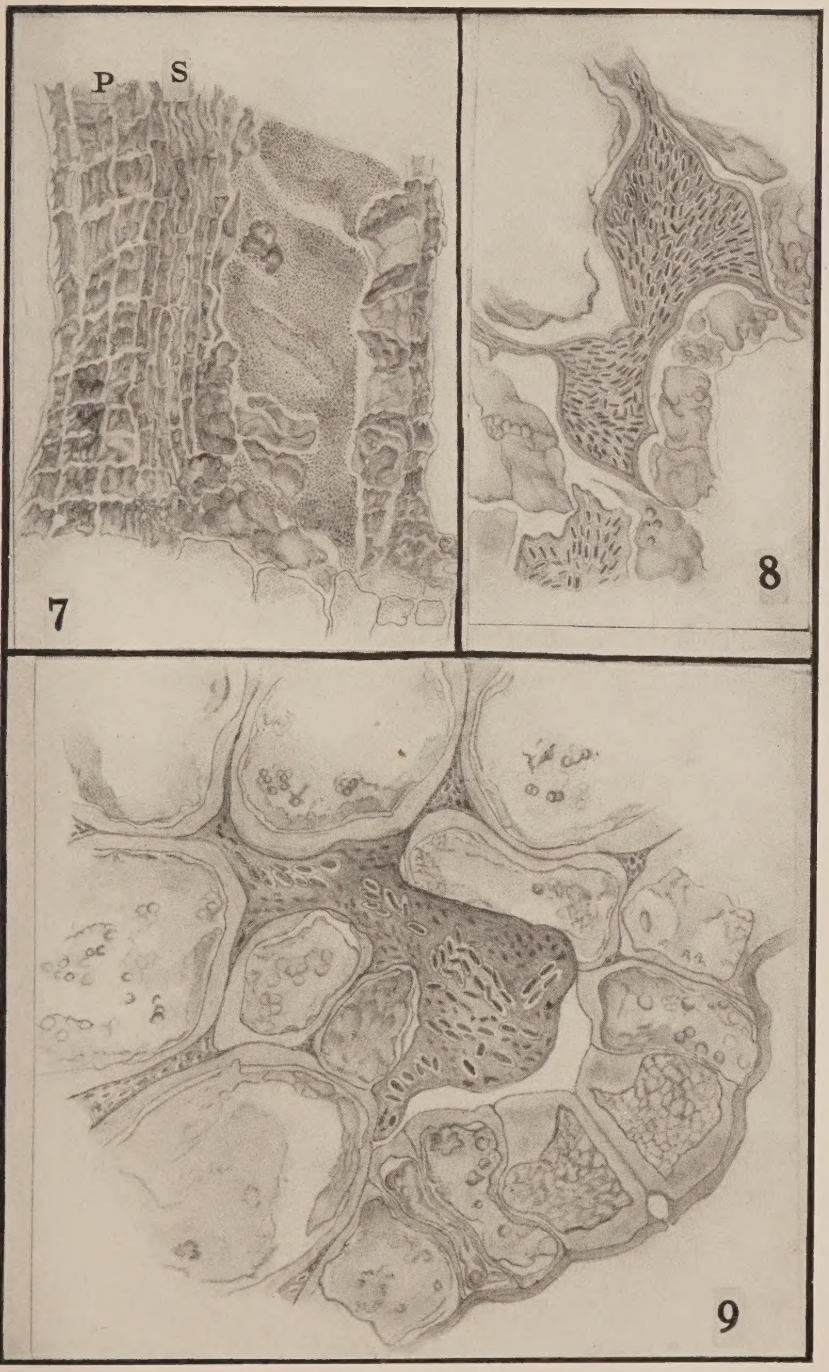
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